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CYTOLOGY, HISTOLOGY. AND PATHOLOGY OF PLENODOMUS MELILOTI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cytology, Histology, and Pathology of Plenodomus meliloti" submitted by Hans John Netolitzky in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A survey for brown root-rot of legumes caused by Plenodomus meliloti was conducted to determine the prevalence of the disease. In central Alberta little disease damage was evident but the disease was very extensive in the Yukon Territory.

Pathogenicity studies were carried out and P. meliloti was found to be pathogenic to legume and cereal seedlings grown in potato sucrose agar. Infection from inoculation of greenhouse and field plants was not successful. The effects of a fungus toxin was demonstrated.

The development of pycnidia and discharge of pycnospores were studied using histological techniques. Pycnidia were found to be initiated as a result of simple meristogenous growth and consisted of a pseudoparenchymatous mass in the center of which a locule formed. Conidiophores 5-10 μ in length developed on the periphery of the locule. Spores formed at the tip of the conidiophores and were discharged through beaks approximately 60 days after pycnidia were initiated.

Cytological studies showed that, in general, the mycelium was multinucleate, pycnospores were uninucleate, and the haploid chromosome number was found to be three.

The fungus grew vigorously on potato-sucrose agar, malt-yeast agar, corn meal agar, sterilized stems of alfalfa and sweet clover but to a lesser extent on plant extracts and living sweet clover roots immersed in kinetin solutions.

The effects of temperature on P. meliloti were determined and it was found that various isolates grew well at 5, 10 and 20° C. Field and laboratory experiments indicated that the fungus would survive extremely low temperatures.

ACKNOWLEDGEMENTS

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INTRODUCTION

The fungus Plenodomus meliloti Mark.-Let. has been reported from a number of widely separated areas. It was first reported in the U.S.S.R., near Leningrad by Markova-Letova (33) in 1927, from Alberta and Saskatchewan in 1930 by Dearness and Sanford (11), in 1955 from Manitoba by McDonald (34), in 1958 from Alaska by Lebeau and Logsdon (31), and recently was reported in Finland by Salonen (41). Markova-Letova (33) described and named this organism Plenodomus meliloti Mark.-Let. in 1927. Sanford (42) reported a root rot of legumes in 1926, but an official description of the incitant was not published until 1930 (11). According to the International Code of Botanical Nomenclature, 1956 (29), priority in naming this organism should be given to Markova-Letova. McDonald (34) in his study of fungi rotting alfalfa roots refers to this organism as P. meliloti Mark-Let. (= P. meliloti D. and S.). Dickson (14) refers to this fungus as P. meliloti Mark.-Let.

This organism has been reported on a number of hosts including Medicago, Melilotus, Axyris amaranthoides, Amaranthus retroflexus, and Avena sativa (43). Robertson (38) isolated P. meliloti from hollyhock (Althaea rosa) and Lebeau and Logsdon (31) observed this organism on Festuca rubra and Poa pratensis.

The disease symptoms as described by Sanford (43) are characterized by brown, slightly sunken, necrotic lesions, which appear on the tap or lateral roots of sweet clover, alfalfa, and common clover (Trifolium pratense). Any part of the root may be affected, although

the tips of smaller roots succumb first. In these lesions are embedded numerous pycnidia. Sometimes only a few or even no pycnidia are observed.

Although Dearness and Sanford (11) and Cormack (8) described the fungus, details of pycnidial formation and discharge of spores are lacking. Pathogenicity studies were carried out by Sanford and Cormack (44), Cormack (8), and McDonald (34) to determine whether P. meliloti was a primary pathogen; however, their results were inconclusive. For these reasons studies were initiated to elaborate on the morphology and pathogenicity of P. meliloti. Since little environmental and no cytological studies have been carried out on this fungus, these aspects were included in the investigations.

DISTRIBUTION OF BROWN ROOT ROT

Alberta

In the early part of June 1963 surveys were made to determine the prevalence and distribution of P. meliloti in central Alberta. The areas included in this survey were Edmonton, Morinville, Westlock, Barrhead, Sangudo, Lacombe, and Rocky Mountain House. In each of these surveys lesions resembling those caused by P. meliloti were found; however, pycnidia were observed only in the Edmonton and Lacombe areas.

Yukon Territory

In May of 1963 diseased plant material was collected by Dr. Colotelo in the vicinity of the Dominion Experimental Farm, Mile 1019, Yukon Territory. Plants found to be infected by P. meliloti were yellow sweet clover, red clover, alfalfa, white Dutch clover and naturally occurring vetches and lupins.

Numerous pycnidia were present on the roots of these diseased plants (Fig. 1). The pycnidia, in some cases, completely covered the diseased portions of the root. In many cases both lateral and tap roots were missing.

The following year, 1964, two surveys were made by the author at the Dominion Experimental Farm, Mile 1019, Yukon Territory. The first was conducted in the early part of June, and the second in September. Again, pycnidia of P. meliloti were found in large numbers on the roots of alfalfa, and sweet clover. Pycnidia resembling those

Fig. 1. Pycnidia of P. meliloti on the root of Grimm alfalfa obtained in the Yukon Territory.

of P. meliloti were also observed on Olli barley and dead roots of cabbage. The native lupin, Lupinus arcticus, showed root damage, characteristic of that caused by P. meliloti on cultivated legumes. Numerous pycnidia were observed on the lower parts of the stems (Fig. 2) and crowns. Other indigenous flora examined included Poa spp. and other grasses. No symptoms and/or signs of P. meliloti were found.

In the second survey, made in September, there was a great reduction in the number of pycnidia. Many lesions, similar to those of brown root rot of legumes, were observed on the roots and crowns of legumes. Pycnidia were found mainly on the crowns and small lateral roots. A large number of pycnidia were observed on the roots of reed canary grass (Phalaris arundinacea).

From June to October 1963, plant materials which included legumes and cereals, were obtained from the Dominion Experimental Farm, Mile 1019, Yukon Territory, at intervals of two weeks (by air express). This procedure was followed in 1964 except that plants were sent earlier, beginning in May.

From these samples it was noted that there was a rapid increase in the number of pycnidia on roots of various hosts in the early spring shortly after the soil temperature rose above freezing. During late spring and early summer there was a gradual reduction in the number of pycnidia and lesions. Pycnidia on roots of Medicago falcata, which were sent during mid-summer, were oozing spores on arrival. In September very few pycnidia were found on the roots but there was a slight increase in the number of pycnidia on the crowns and upper portions of the root.

Fig. 2. Pycnidia of P. meliloti on lower stem of Lupinus arcticus.

Discussion

The results of the disease surveys indicate that P. meliloti does not seriously affect legumes in central Alberta to the same extent that it does in the Yukon Territory.

From material obtained from the Yukon Territory it was concluded that infection occurred the previous fall and perhaps, to some extent, during the winter.

Although pycnidia were observed to ooze spores in late summer, it is reasonable to assume that discharge of spores takes place from spring until late summer. These spores would therefore constitute the inoculum and result in the infection of various hosts.

The rapid increase in the number of root lesions and pycnidia in spring and their decline in the summer agrees with the observations of McDonald (34) in Manitoba. He found that P. meliloti comprised 30 per cent of the fungi isolated in the spring from alfalfa roots; however, by mid-summer, the occurrence of P. meliloti had fallen to two per cent and by autumn it was three. McDonald does not indicate whether pycnidia were observed.

ISOLATES AND THEIR ORIGIN

A. Isolation of Mycelium

Materials

Diseased plant material was collected in the vicinity of the Dominion Experimental Farm, Mile 1019, Yukon Territory, Edmonton, and the Dominion Experimental Farm at Lacombe.

Isolates used in these studies were numbered; their origin and the hosts from which they were isolated are as follows:

Isolates	Origin	Hosts
P_7, P_8 & P_{10}	Alberta and Saskatchewan	legumes (varieties unknown)
P_{15}	Edmonton	*Melilotus* sp.
P_{20}	Mile 1019, Alaska Highway	*Medicago* sp.
P_{30}	Finland	*Trifolium pratense*
P_{40}	Mile 1019, Alaska Highway	*Brassica oleracea* var. *capitata*
P_{50}	Mile 1019, Alaska Highway	*Lupinus arcticus*
P_{60}	Mile 1019, Alaska Highway	*Phalaris arundinacea*
P_{70} & P_{71}	Mile 1019, Alaska Highway	*Oxytropis campestris*

Isolates P_7, P_8, P_{70}, and P_{71} were obtained from the Plant Pathology Laboratory, Canada Department of Agriculture, Lethbridge, Alberta, P_{10} from the Bureau of Schimmelcultures, Baarn, Netherlands and P_{30} from Dr. A. Salonen, Finland. The remaining isolates P_{15}, P_{20}, P_{40}, P_{50}, and P_{60} were isolated by the author.

Methods of Isolation

Two methods were employed.

1. Diseased roots of yellow sweet clover, red clover, white clover, lupin, vetch, and trefoil were washed free of all soil with tap water, cut into small pieces, again washed with water, surface sterilized by dipping in 0.1 per cent mercuric chloride for 5 seconds, and then thoroughly rinsed in sterile water to remove traces of mercuric chloride. These sections were aseptically placed in Petri plates containing malt-yeast agar (M.Y.A.). Lactic acid was added to several Petri plates containing M.Y.A. to prevent bacterial growth. Care was taken to ensure that each section of tissue was in good contact with the medium. The Petri plates were cultured in the dark at 15° C. When fungi appeared around the tissue sections, mycelium was transferred into Petri plates and test tubes containing M.Y.A.

2. Pycnidia removed from diseased alfalfa and sweet clover roots were placed in a small plastic funnel modified for washing pycnidia. Pycnidia were washed with tap water for 2-3 hours, placed aseptically on acidified M.Y.A., and cultured in the dark at 15° C. The resulting mycelium was subcultured.

Results

Fungi isolated by the first method included, Fusarium, Penicillium, Rhizopus, yeasts, and unidentified fungi. P. meliloti was not identified. This method proved to be very unsatisfactory because of the large number of organisms isolated. Probably P. meliloti

was isolated but its growth masked or inhibited by the other organisms. Ylimaki (50) reports 350 fungal isolates from diseased roots of red clover.

The second method proved to be very satisfactory, producing very few fungi other than P. meliloti. Little difficulty in obtaining the mycelium of the isolates of P. meliloti was encountered.

B. Single-spore Isolation

Materials and Methods

Spores of P_{20}, discharged from a pycnidium, were placed aseptically in sterile water. An aliquot of spore suspension was placed in a Petri plate containing potato-sucrose agar (P.S.A.) and incubated for 72 hours in the dark at 15° C. A Fonbrune micromanipulator was used for single-spore isolation (20).

Results

All the resulting single-spore cultures, grown on P.S.A., appeared similar to the original culture. Sub-cultures of these single-spore isolates were subsequently used for further studies.

CYTOLOGY

Literature Review

Feulgen and Rossenbeck (19) in 1924 introduced the Feulgen technique as a specific stain of the nucleus. This stain has become universally considered the most reliable cytological stain for desoxyribonucleic acid (DNA).

Stacey et al. (45) reported that with relatively gentle acid hydrolysis, desoxyribose of DNA was transformed into w-hydroxy laevulinic aldehyde. It is this aldehyde which is responsible for the blue coloration in the Feulgen test.

The Giemsa stain, as introduced by Giemsa (21), and modified by Robinow (39) to HCl-Giemsa, was investigated by Jacobson and Webb (26). The Giemsa stain, a complex mixture of eosin with methylene blue and its oxidation products, is an amphochrome stain containing both mordant and dye in one solution. Jacobson and Webb report the color reaction of Giemsa, in intact cells and for isolated nucleoproteins, differs between ribonucleic acid (RNA) and DNA, RNA staining red and DNA blue. As a result, a good differentiation between cytoplasm and nucleus can be obtained.

Materials and Methods

Isolates

Isolates used in staining with HCl-Giemsa were P_7, P_8, P_{10}, P_{15}, P_{20}, P_{70}, and P_{71}. Only isolate P_{20} was stained with Feulgen's reagent.

Staining Procedures

The principal nuclear stains used in these studies included Feulgen (19) and HCl-Giemsa (21), however, propionocarmine (32) and Heidenhain's Iron Hematoxylin (27) were also used.

The preparation of Feulgen and HCl-Giemsa stains are given in the Appendix.

Feulgen

Spores of P_{20} were germinated in sterile water or in a synthetic liquid medium (47). Germinated spores were removed at various stages of development, placed on microslides, air dried and stained. The Feulgen staining procedure is as follows:

- fixative: absolute ethanol : glacial acetic acid (3 : 1)	15 min.
- hydrolysis, in 1 N HCl at 60° C.	
- mycelium	6 min.
- spores	4 min.
- Feulgen	1 hr.
- 20% ethanol	5 sec.
- 95% ethanol	5 sec.
- absolute ethanol (two changes)	5 sec. each

HCl-Giemsa

The isolates to be stained were grown on microslides coated with a thin film of medium. The inoculum consisted of a plug of actively growing mycelium (4 mm. dia.) and spores of P_{20}.

In this procedure microslides were sterilized with absolute ethanol and the ethanol removed by flaming. Media included a synthetic medium (47), M.Y.A., and P.S.A.

The slides, coated with medium, were placed aseptically in Petri plates containing moistened filter paper and on pieces of V-shaped glass tubing. Isolates were cultured in the dark at 15° C. until the diameter of fungal growth was about 1 - 1.5 cm. At this time the plug used for inoculation was removed and the microslides were air-dried. The procedure used for staining with Giemsa is as follows:

- Carnoys fixative	10 min.
- 95% ethanol	5 - 10 sec.
- 75% ethanol	5 - 10 sec.
- H_2O	10 - 15 sec.
- 1N HCl cold	5 min.
- 1N HCl (60° C)	5 - 5.5 min.
- washing successively through 5 jars of H_2O	5 min. total
- Giemsa	1 - 2 hr.
- distilled H_2O	5 sec.
- phosphate buffer	5 min.
- mount in abopon (Valnor Corporation)	

Results

No difference in the number and distribution of nuclei in mycelium was observed. Nuclei stained with HCl-Giemsa gave excellent results when synthetic medium was used as the growth medium. However,

with P.S.A. and M.Y.A. the results were unsatisfactory, because the media produced a pink-colored background on the microslide and this was found unsuitable for critical microscope work.

The number and distribution of nuclei in mycelium of isolates stained with HCl-Giemsa and Feulgen were similar. Spores contained, as a rule, one nucleus (several contained 2 or 3 nuclei); hyphal tips contained numerous nuclei; young mycelium from 1-6 nuclei (Fig. 3 - a,b,c,d,e).

Older mycelium stained with HCl-Giemsa, contained 1 to 20 nuclei, (Fig. 3-e) average 4 per cell; however, with Feulgen's reagent, nuclei were not stained because of difficulties encountered during the transfer of older mycelium from culture medium to microslides.

Mycelium of the P_{20} single-spore isolate, stained with HCl-Giemsa, showed no difference in number or distribution of nuclei from that of the other isolates.

The propionocarmine squash technique and Heidenhain's Iron Hematoxylin were not successful.

The chromosome number for spores and mycelium was three (Fig. 4 and 5).

The Giemsa preparation of chromatin material stained more intensely and produced sharper and larger outlines of nuclei and chromosomes than Feulgen preparations. This agrees with Robinow's work (39) for bacteria.

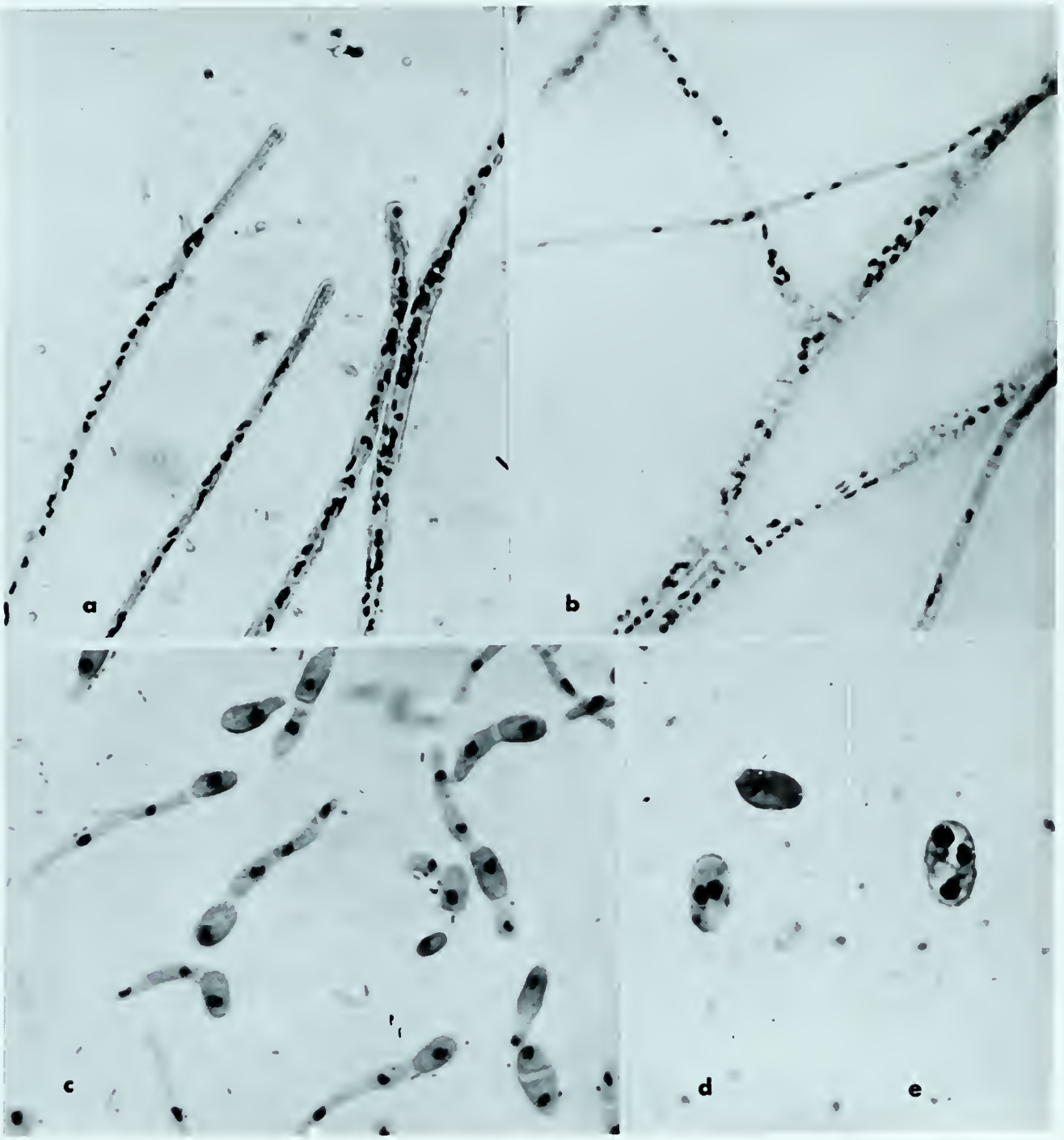


Fig. 3 (a,b,c,d,e). Nuclei of mycelium and pycnospores of P. meliloti, isolate P_{20}, stained with HCl-Giemsa.

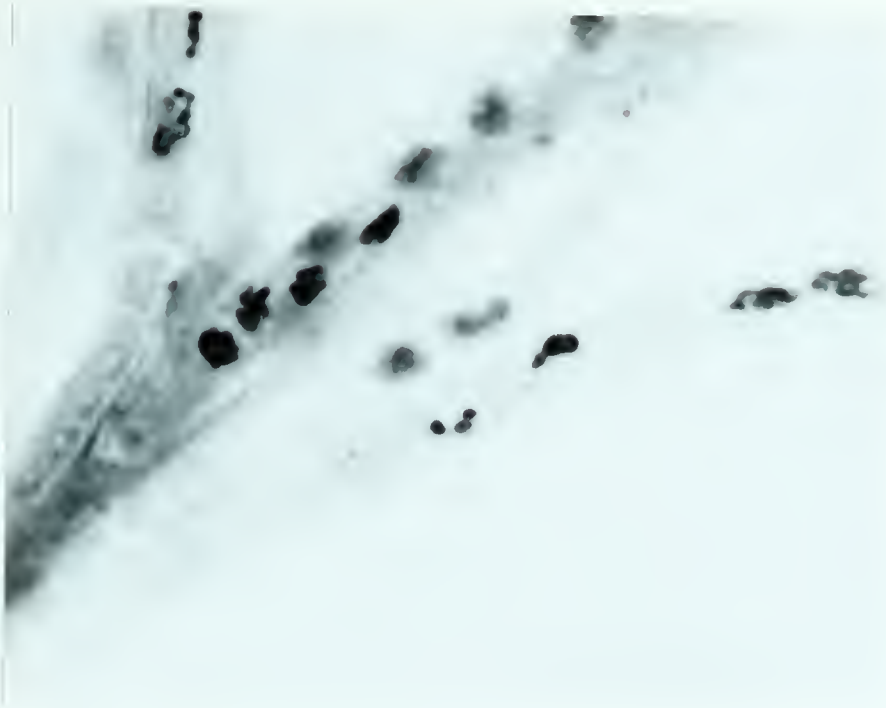


Fig. 4. Chromosomes of P. meliloti, isolate P_{20}, in mycelium. Stained with HCl-Giemsa.

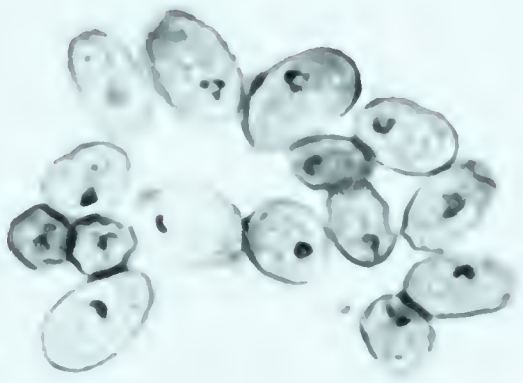


Fig. 5. Chromosomes of P. meliloti, isolate P_{20}, in spores. Stained with Feulgen reagent.

Spores germinated at one or at both ends. After the nucleus divided, one of the newly formed nuclei moved into the germ tube. Nuclei have been observed to divide prior to the formation of a germ tube(s). Subsequent to the production of germ tubes in the initial stages of growth, the mycelium was uninucleate becoming multinucleate as the mycelium matured.

In connection with these studies, hyphae have been observed to anastomose.

Discussion

Variability in the numbers of nuclei in hyphal segments, hyphal branches within each isolate, as well as anastomoses gives rise to the possibility of genetic variation. This may influence the pathogenicity and growth of the fungus. The extent of this variability has not been investigated.

The occurrence of P. meliloti on a large number of hosts, in the parastic and saprophytic stages, indicates wide adaptability.

PYCNIDIA FORMATION

Literature Review

Characteristics which place fungi in the genus Plenodomus, described by Preuss in 1884, are listed by Saccardo (40) and Diedicke (15). With the description many seemingly unrelated fungi can be placed in this genus. For example, Harter (24), when describing P. destruens, the incitant of foot-rot of sweet potato, states that pycnidia do not meet all the requirements of the genus as laid down by Saccardo (40) and Diedicke (15). Harter believes that it is better to place this organism in Plenodomus where it appears to fit better than in any other genus, rather than to form a new group. Groves (23) states that there is considerable confusion in the classification of fungi either in Plenodomus or Phoma. He feels that changes in environment are responsible for changes in morphological development. This may very well be true since the isolate of P. meliloti, isolated by Dearness and Sanford in 1926, and recently obtained from the Bureau of Schimmelculture, Netherlands, has lost its capacity to produce pycnidia (3).

Prior to these studies a detailed description of conidiophores has not been presented. Those presented by Markova-Letova (33), Dearness and Sanford (11), and Salonen (41) are not complete. In general, they state that conidiophores are hyaline, simple, unbranched, short or obsolete. Although no measurements or illustrations are presented, Markova-Letova (33) does state that conidiophores are not longer than spores.

Materials and Methods

The isolates used in this study included P_{15}, P_{20}, and P_{30}. A culture of isolate P_{10} submitted to the Bureau of Schimmelcultures in 1934 by Dearness and Sanford (3) could not be included because it no longer produced pycnidia. Pycnidia used for these studies were obtained from cultures grown in the dark at 15° C. on P.S.A. and M.Y.A. media.

Pycnidia of the developing fungus were removed at intervals and fixed in Rawlin's fixative (see Appendix for preparation). To obtain rapid penetration of the pycnidia with fixative, vials containing pycnidia and fixative were placed under reduced pressure. Pycnidia were stored for a minimum of 48 hours in fixative prior to dehydration with tertiary butyl alcohol, as described by Johansen (27). Following dehydration, the material was placed in tertiary butyl alcohol and paraffin (3:1) for 2-4 hours and then into tertiary butyl alcohol and paraffin (1:1) for 2-4 hours. The material was then infiltrated with paraffin (sealing wax) over night and finally embedded with Fisher's Tissuemat (M.P. 62.5° C.).

The embedded material was cut into sections of 6, 8, 10, and 12 microns (usually 10 or 12 microns) in thickness with a Spencer rotary microtome. The resulting ribbons were mounted on glass microslides with Haupt's adhesive (27) and air-dried for at least 12 hours.

Sections were stained with safranin and counterstained with fast green. Several pycnidial sections were treated with Heidenhain's Iron Hematoxylin (27) nuclear stain.

The staining procedure for safranin and fast green is as follows:

- xylene	10 min.
- xylene-absolute ethanol (1:1)	5 min.
- 100% ethanol	1-2 min.
- 95% ethanol	1-2 min.
- 85% ethanol	1-2 min.
- 70% ethanol	1-2 min.
- safranin in 70% ethanol	4-5 min.
- 70% ethanol	1-2 min.
- 85% ethanol	1-2 min.
- 95% ethanol	1-2 min.
- fast green in 95% ethanol	5 sec.
- 95% ethanol	1-2 min.
- 100% ethanol	1-2 min.
- xylene-absolute ethanol (1:1)	5 min.
- xylene	5 min.

Results

The results for P_{15}, P_{20}, and P_{30} will be discussed together. Although a description, which included photographs of pycnidial development and spore discharge of P. meliloti has been presented by Colotelo and Netolitzky (6), an account will also be given here.

Pycnidia appeared on P.S.A. within 8-10 days after inoculation. They developed as a result of simple meristogenous growth. In this

process several cells of coarse or mature hyphae divide to form smaller swollen cells (Fig. 6-a). These in turn divide longitudinally and transversely to form a pseudoparenchymatous mass (Fig. 6 - b,c,d,e). This mass enlarges greatly and is generally entangled in the mycelium. The periphery of this mass becomes a periderm consisting of dark heavy-walled sclerenchymatous cells.

Locule formation, within the parenchymatous mass, was first observed when pycnidia were 30 days old (Fig. 7-a). The region in which the locule formed was found to stain a blue-green color in contrast to the remaining tissues which stained green. Cells in the locular region become mucilaginous due to lysigenetic action (Fig. 7-b). Conidiophores bearing conidia were observed on the periphery of the locule. Intermediate stages in the formation of conidiophores were not observed. Conidiophores were found to be unbranched and consisted of a bulbous or inflated base from which extended a long filament and varied in length from 5-10 μ in the same pycnidium (Fig. 8). Details showing conidia and conidiophores have been presented in the form of a camera lucida drawing by Netolitzky and Colotelo (35).

With the microscope, the conidiophores were extremely difficult to observe under normal bright-field illumination. They were best observed with phase-contrast illumination after being treated with Heidenhain's Iron Hematoxylin just after the first conidiophores were formed. As the pycnidia matured the numbers of pycnospores were so large that conidiophores were not seen.

As the number of pycnospores increased to fill the locule, a breakdown of an avenue of cells leading from the locule to the end

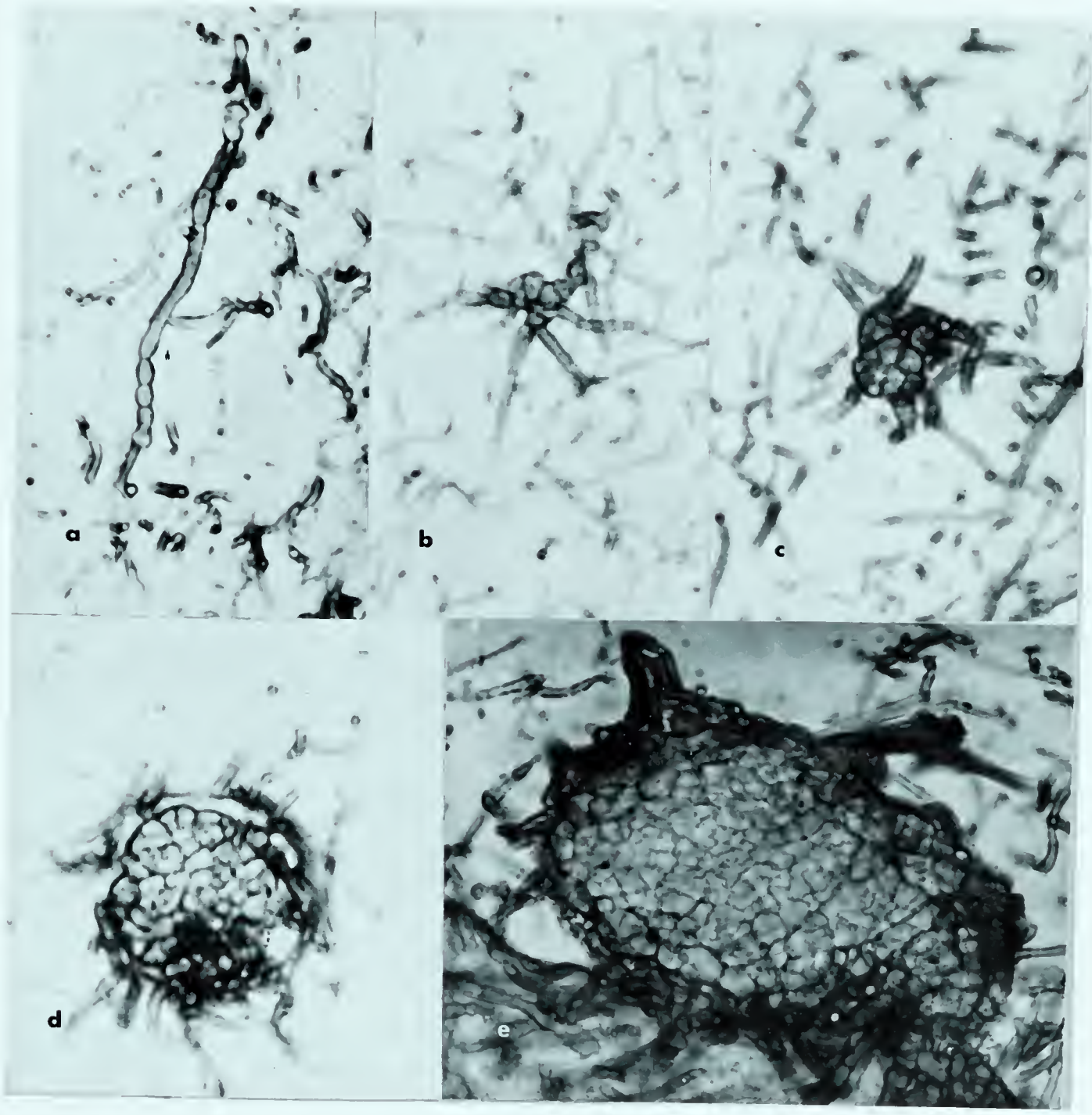


Fig. 6 (a,b,c,d,e). Pycnidial development of P. meliloti on P.S.A.
(a) Early stage of meristogenous development showing short swollen cells of mycelium.
(b,c,d,e) Stages in the formation of pseudoparenchymatous mass.

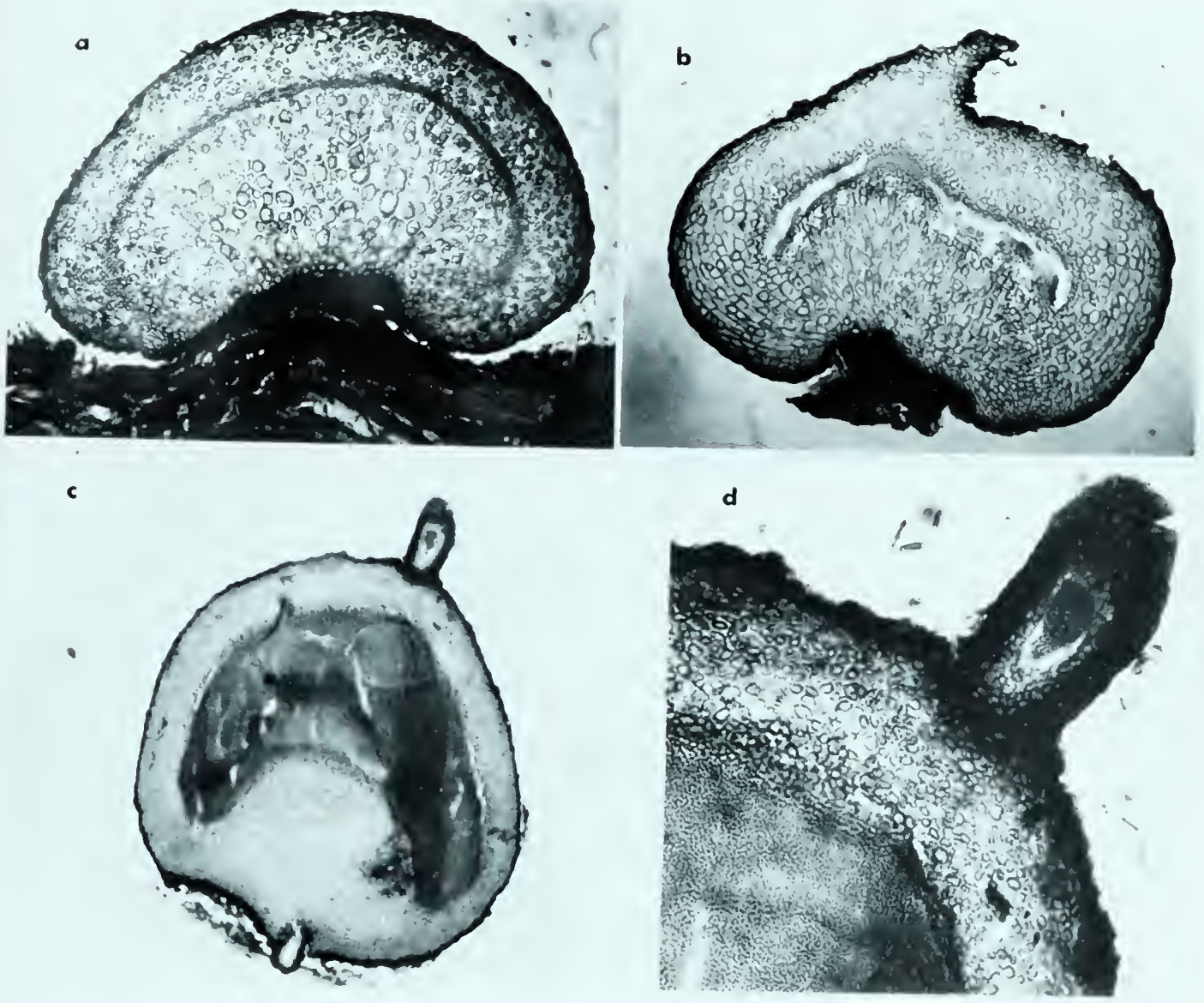


Fig. 7 (a,b,c,d). Pycnidial development of P. meliloti on P.S.A. (continued).
(a) Early stage in the formation of a locule.
(b) Pycnidium with locule in an early stage.
(c) Mature pycnidium with locule and beak containing pycnospores.
(d) Enlarged view of (c) showing pycnospores in beak.

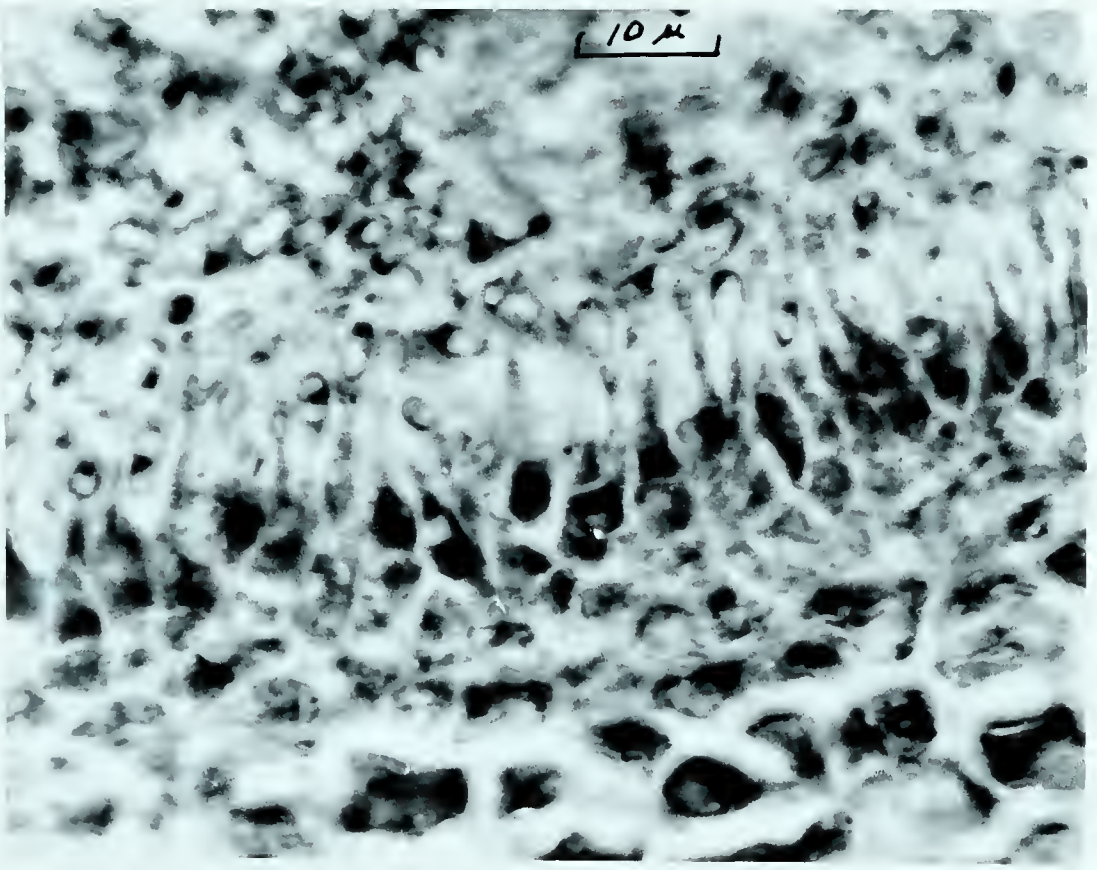


Fig. 8. P. meliloti, isolate P_{20} - conidiophores bearing conidia.

of the beak occurred to form a channel. Upon discharge, pycnospores were forced along this channel and through the ostiole which formed at the tip of the beak (Fig. 7-c,d, and 9-a).

Soon after or during locule formation single or branched tubular beaks of various lengths developed from the peridial wall (Figs. 7-d, and 10). The morphology of the beaks was similar to that of the main body of the immature pycnidium, i.e. pseudoparenchymatous tissue surrounded by periderm made up of dark sclerenchymatous cells (Fig. 9-b). Occasionally the end of the beak was bulbous and this disappeared upon spore discharge or when touched with a sharp object.

Spores were discharged as a cream to yellow globular mass approximately 60 days after pycnidia were initiated (Fig. 9-c,d). Occasionally pycnospores were discharged from papillate openings in the peridial wall.

Spore discharge of pycnidia grown in culture was found to be triggered by an increase in temperature and/or the addition of water to the culture medium.

Discussion

Pycnidia of *P. meliloti* appear to follow the pattern of pycnidial development described by De Bary (12) for *Pleospora polytricha* and by Kempton (28) for species of *Phoma* and *Macrophoma*. In most cases the pycnidial primordia of *P. meliloti* are initiated by meristogenous development which is the segmentation of several cells of single hyphae into a parenchymatous mass rather than a tangle of hyphae entwined into a knot which is known as symphogenous development.

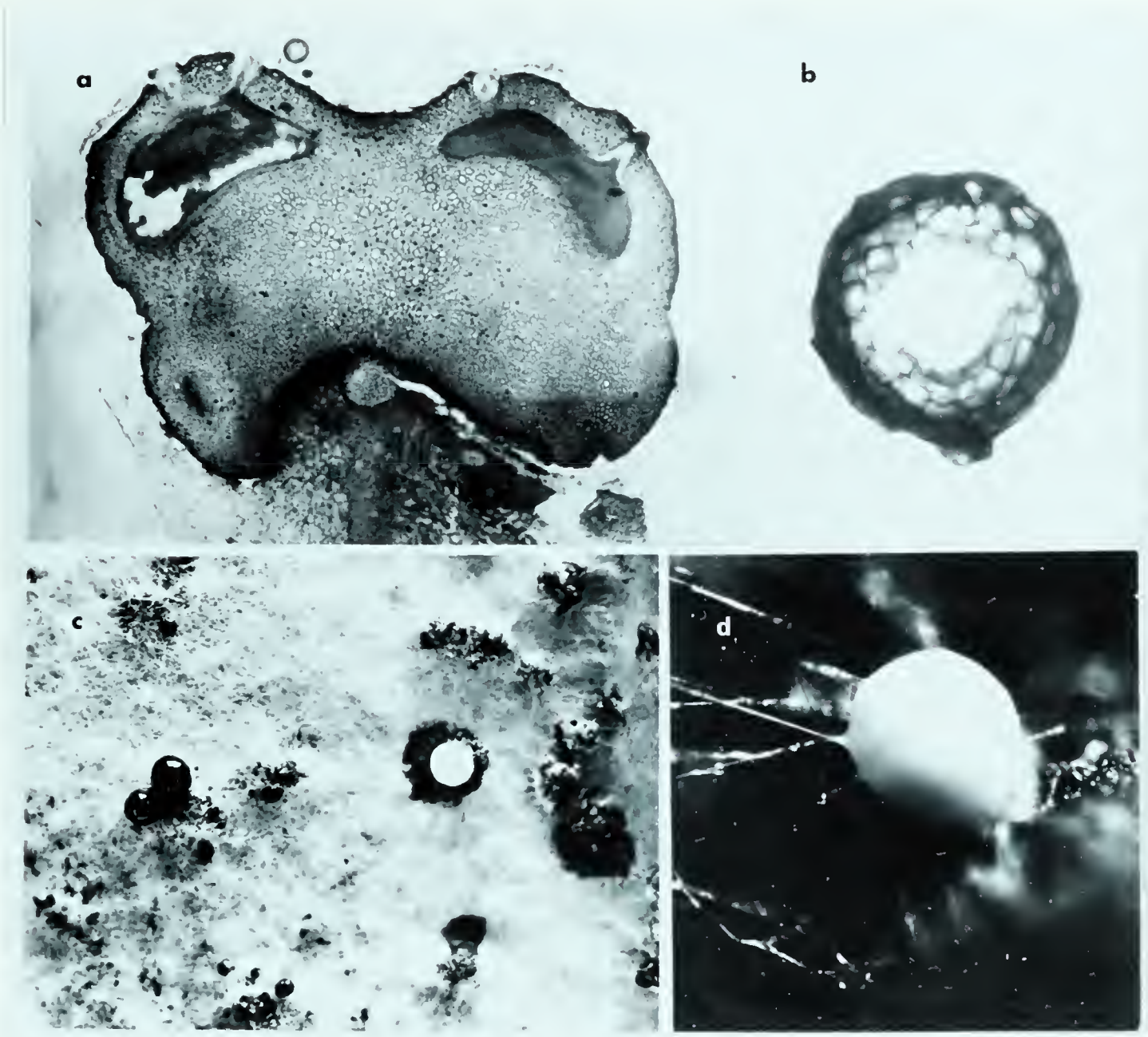


Fig. 9 (a,b,c,d). Discharge of pycnospores from pycnidia of P. meliloti grown on P.S.A.
(a) Pycnidium with two locules discharging pycnospores.
(b) Transverse section of beak.
(c) Exudates and pycnospores being discharged from pycnidia.
(d) Pycnospores being discharged from beak.

Fig. 10. Pycnidia of P. meliloti showing various forms of beaks.

The formation of locules and the passage leading from the locule to the tip of the beak was formed as the result of lysigenetic activity. This process of cavity formation has been studied in detail by Dodge (16) for a number of fruit pathogens.

The results presented here agree with those of Sanford (43) and Salonen (41) who also found pycnidia with one or more spore-bearing chambers and several ostioles. Salonen (41) found that spore discharge occurred 60-80 days after culture growth terminated. What Salonen interprets by termination of growth is not defined. Sanford (43) found that spores formed when pycnidia were 50 days old and the spores released 30-50 days later. In this study spores were released as early as 60 days after inoculation. Sanford (43) and Salonen (41) probably used different cultural techniques and this may account for the differences in results.

Conidiophores, as measured in these studies, were found to vary considerably in their length. The observations of Markova-Letova (33) that conidiophores were not longer than spores may be due to the inherent properties of her isolate.

CULTURAL STUDIES

Introduction

P. meliloti was grown on a number of media, and on host tissue in order to study the characteristics of the fungus and to determine a suitable medium upon which the fungus could be subcultured.

Also it was hoped that the use of a wide variety of media would result in the production of the perfect stage. It is known that many fungi require specific nutritional requirements before the production of a sexual stage (25).

Materials and Methods

A. Growth on Various Media

Isolate P_{20} was grown on P.S.A., M.Y.A., Sach's, corn meal agar and plant extracts.

Plant material for the preparation of the plant extracts included roots, stems, a mixture of leaves and stems of sweet clover, and alfalfa roots.

The procedure used for the preparation of plant extracts is as follows: Alfalfa and sweet clover roots (approximately 140 grams fresh weight) were washed in tap water and autoclaved in 1000 ml. of demineralized water for 1 hr. at 120° C. After two days the material was again autoclaved for 1 hr. This was homogenized using a Waring blendor, and demineralized water was added to facilitate homogenization.

The homogenate was expressed twice through double layers of cheesecloth and the pulp discarded.

The orange-brown liquid was filtered through Eaton-Dikeman #615 and #617 filter papers to further remove particulate root matter. The volume of the filtrate was reduced to 250 ml. by boiling. Further clearing of this root extract was obtained by centrifuging at 9000 r.p.m. (10,000 x g.) for 10 min. using a Servall Superspeed Centrifuge. Difco agar (1.7%) was added and sterilized by autoclaving at 120° C. for 20 min. Petri plates containing this medium were inoculated with mycelium of P_{20} and cultured at 15° C.

The above procedure was repeated using roots, stems, a mixture of leaves and stems of Arctic and Cumino sweet clover. In this procedure different weights of material were used; however, the final volume of the extract was adjusted so that the ratio of the final volume to fresh weight used was approximately 2:1.

Half of each sample was stored at -12° C. and the other half was freeze-dried to a powder form since it was thought that liquid extracts, although frozen, would deteriorate in a short period of time during storage. P.S.A. was used as the control medium for comparison.

The results are presented in Table I (see page 33).

B. Growth on Host Tissue

It was thought desirable to grow the fungus on detached sections of root and stems of alfalfa and sweet clover. These materials would be more closely related to that which the fungus would encounter

under natural conditions than media used in previous studies, e.g. P.S.A., M.Y.A., and corn meal agar.

This was carried out by (a) placing autoclaved sections of host tissue on water-agar medium and (b) live sections were partially immersed in kinetin solutions.

(a) Growth on autoclaved host tissue

Sections of roots and stems (3-4 cm. in length) of alfalfa and sweet clover were washed in tap water and sterilized by autoclaving for 1 hr. at 120° C. These pieces were placed aseptically in Petri plates containing water-agar medium, inoculated with mycelium of P_{20}, and incubated in the dark at 15° C.

(b) Growth on living tissue

Sweet clover roots were washed in tap water for 35 min. and cut into lengths of 2-3 cm. Several sections were split in half, longitudinally, washed again in tap water for 10 min. and placed in Petri plates containing sterile water and kinetin. Concentrations of kinetin used included 1, 5, and 10 p.p.m. Four sections were placed in each Petri plate, two vertically and the other two horizontally.

Pycnidia were placed on the upper surface of the vertical section and in a previously notched portion at the ends of the horizontal sections. Other sections were inoculated with mycelium in the same manner.

Care was taken to prevent the transfer of medium with the inocula. Pycnidia were washed with tap water for an hour prior to inoculation to remove adhering medium.

Sections of plant roots placed in sterilized tap water and inoculated, as described above, were used for comparison. All cultures were incubated in the dark at 15° C.

Results

A. Growth on Various Media

Although mycelium of P_{20} grew well on P.S.A., M.Y.A., and corn meal agar, differences in growth were observed. The mycelium on P.S.A. and M.Y.A. was cottony and greyish-white in color. Pycnidia were initiated in about 10 days. On corn meal agar very little aerial mycelium formed but many large pycnidia were produced.

Growth of mycelium on Sach's medium was very poor with no pycnidia being formed.

Droplets of clear to amber liquid were observed to exude from immature pycnidia (Fig. 9-c). This exudate was analyzed by Colotelo and Netolitzky (6) using paper chromatographic techniques, and found to contain 17 amino compounds, but neither reducing sugars nor sucrose were detected.

During the course of these studies it was observed that the pycnidial beaks became progressively shorter when the fungus was continuously subcultured on M.Y.A.

The mycelium of P_{20} used in this study was ash-grey in color on extracts of root, stems and P.S.A., but was fluffier and white on the leaf and stem medium.

Table I

Growth[1] of P. meliloti, isolate P_{20}, on plant extracts and P.S.A.

Medium	Growth of Mycelium	Number of Pycnidia
P.S.A.	10	10
Alfalfa[2] (roots)	7	9
Sweet clover[3] (roots)	7	9
Arctic-stems (frozen)	8	2
Cumino-stems (frozen)	8	2
Arctic-stems (fresh)	8	2
Cumino-stems (fresh)	8	2
Arctic-stems (freeze-dried)	6	1
Cumino-stems (freeze-dried)	6	1
Arctic-leaf and stem (fresh)	6	2
Cumino-leaf and stem (fresh)	6	2
Arctic-leaf and stem (frozen)	3	1
Cumino-leaf and stem (frozen)	4	1
Arctic-leaf and stem (freeze-dried)	6	2
Cumino-leaf and stem (freeze-dried)	6	2

[1] Cultures on plant extracts were rated in relation to those on P.S.A. Cultures on P.S.A. were given a rating of 10 since this medium was most favourable for growth of mycelium and production of pycnidia.
[2] Field collection variety unknown.
[3] Field collection variety unknown (white blossom).

The data of Table I show the growth of mycelium and the production of pycnidia on plant extracts were not as good as on P.S.A.

Although mycelial growth was poor on alfalfa and sweet clover root extracts, the production of pycnidia was high compared to that observed for the other extracts. Production of pycnidia on other extracts was extremely low.

The growth of mycelium on Arctic and Cumino leaf and stem (frozen) extracts was very poor compared to that on other media.

B. Growth on Host Tissue

(a) Growth on autoclaved host tissue

The mycelium grew very rapidly and soon covered the root and stem sections. Pycnidia were observed 14 days after inoculation and within a short period of time, approximately three weeks, completely covered the surface of the stem and root sections (Fig. 11). These pycnidia had very long beaks. Pycnidia also developed on small leaves still attached to stem sections of sweet clover.

The perfect stage was not observed.

(b) Growth on living tissue

Fungal growth on plant sections, partially immersed in kinetin and water, was much inferior to that observed for P.S.A. and M.Y.A., corn meal agar, and autoclaved stem and root sections, however, much superior than on Sach's medium.

Fig. 11. Pycnidia of P. meliloti on autoclaved sections of sweet clover root embedded in water agar.

When mycelium was used as inoculum very little new growth and no pycnidia resulted. However, when inoculum consisted of pycnidia, there was restricted growth of mycelium with a few small pycnidia being formed. Growth of the fungus on sections in kinetin or water was similar. Differences in growth on horizontal and vertical sections were not observed.

The concentrations of kinetin used did not prevent the autolysis of host tissues since tissue sections in kinetin and water showed a yellow discoloration towards the end of the experiment. The experiment was terminated after 28 days because of contamination.

The perfect stage was not observed.

Discussion

It is evident that the commonly used media P.S.A., M.Y.A., and corn meal are able to support the growth of mycelium and the production of pycnidia. The results indicate that corn meal contained some factor(s) which promoted the production of very large pycnidia at the expense of mycelial growth. These results on corn meal agar are similar to those described by Coons (7) for Plenodomus fuscomaculans. He found this medium most favourable for pycnidial production. Coons also found that a medium consisting of filter paper resulted in the growth of mycelium and the production of pycnidia. This form of medium was not used in this study; however, Sach's medium, composed solely of inorganic salts, did not support growth and this would indicate that some form(s) of organic substances is necessary.

The growth of mycelium and production of pycnidia on extracts prepared from a mixture of stems and leaves was poorer than that on autoclaved stems. It is possible that the extraction procedure was severe enough to liberate substances which inhibited the growth of the fungus. Roots which had undergone the same extraction treatment did not inhibit pycnidial formation. Stem sections, which also produced good growth of P. meliloti although autoclaved, did not undergo the severe extraction treatment.

Although autoclaved stems produced good growth of the fungus as did root sections, very little growth of P. meliloti occurs on stems under natural conditions. Several factors may be involved. Probably the most important is that the plant tissue, just above ground level and where the fungus is occasionally found, is very woody and does not offer the same level of nutrition as the roots. Differences in environment of stems and roots may also play a large part in the development of the fungus.

The poor growth on sweet clover leaf and stem (frozen) extracts, in comparison to that on the other media, can not be explained at this time.

Kinetin, at the concentrations used, did not prevent the deterioration of root tissue. It is possible that higher concentrations should have been used. It should also be noted that an exogenous supply of carbon or nitrogen was not added and therefore metabolites used by living host tissue were not replaced. Generally, White's medium (49) is used for growth of excised root tissues and contains these metabolites.

This approach of culturing the fungus on live sections of host tissue may prove fruitful in the future provided some means are taken to eliminate contamination.

TEMPERATURE STUDIES

Introduction

According to the investigations of Sanford (43), the temperature limits of growth for his isolate were 0° C. and 27° C. with the optimum being 15-17° C.

It was thought desirable to compare the effect of various temperatures on the growth of isolates of P. meliloti obtained from various sources (see page 8).

Many fungi are known to survive very low temperatures. Wellman and Walden (48) have shown that of 50 species of fungi tested for viability, after being kept in liquid nitrogen (-196° C.) for 72 hours, 47 survived. Survival was determined by the initiation of new growth. Carmichael (4) reports that of 400 cultures, representing 65 genera, stored for nine months at -20° C. only 17 cultures were lost. Ezekiel (17) showed that Phymatotrichum omnivorum did not survive when kept at a temperature of -13° C. for more than 24 hours. Conidia of Endoconidiophora fragacearum do not survive at temperatures lower than -42° C. for 10 minutes (5).

As no information was available regarding the effects of low temperature on the survival of P. meliloti, a study was undertaken.

A. Growth Rates of Various Isolates

Materials and Methods

Inoculum in the form of plugs (4 mm. dia.), obtained from the outer edge of cultures, was placed mycelial side down on P.S.A. in Petri plates.

Cultures of isolates P_{10}, P_{15}, P_{20}, and P_{30} were incubated at 5, 15, 20, and 25° C. Each treatment was replicated 10 times and the diameters of colonies measured every 48 hours.

Results

Isolates grew vigorously at all temperatures studied except at 25° C. where growth was negligible.

The amount of radial growth for the isolates at various temperatures is shown in Figure 12.

The radial growth of P_{30} was greatest and that of P_{10} the lowest at all temperatures.

The growth of P_{15} and P_{20} was very similar, being intermediate to P_{30} and P_{10}.

B. Effects of Low Temperatures

Materials and Methods

Mycelium, pycnidia, and pycnospores of isolate P_{20} were used in (a) field and (b) laboratory studies.

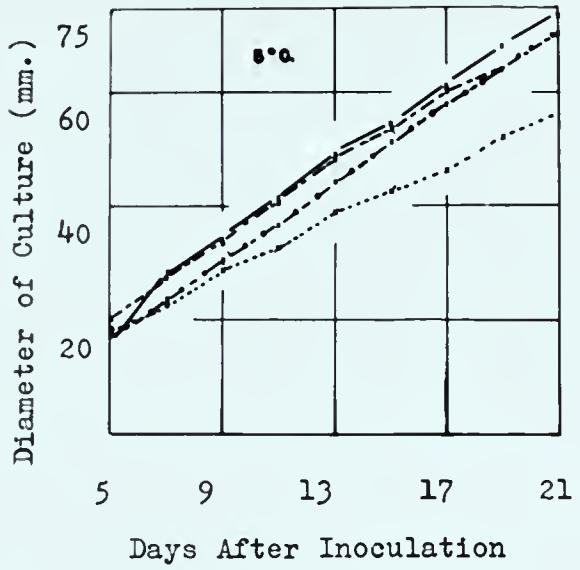


Fig. 12 Growth rates of P. meliloti, isolates P_{10}, P_{15}, P_{20}, and P_{30} at 5, 15, and 20°C.

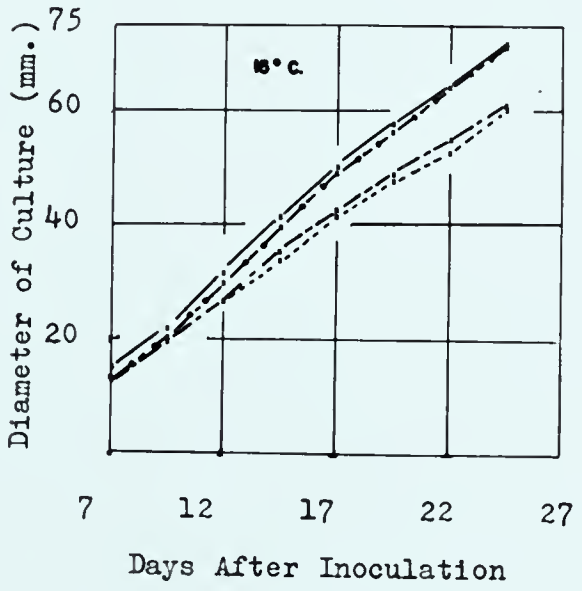
Legend

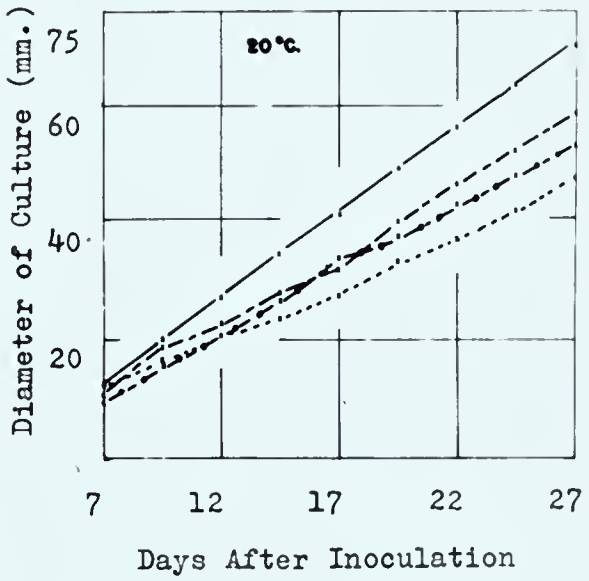
P_{10} - - - -

P_{15} — - —

P_{20} —●—●—

P_{30} ———





(a) Field studies

Mature pycnidia, containing spores, were placed in the soil at a depth of 10 cm. at the University Parkland Farm in October 1963. The soil temperature was recorded at a depth of 10 cm. by dial thermometers. Temperatures were taken at approximately 8:00 a.m. each day except Saturday and Sunday, but in periods of very low temperatures daily readings were made.

Pycnidia were removed periodically beginning in December and the per cent of spores germinating determined. The last sample was removed in March of 1964.

Soil temperatures taken at Mile 1019, Alaska Highway, during the winter of 1963-1964 were compared with those taken at the University Parkland Farm.

(b) Laboratory studies

(i) Pycnidia

Cultures containing mature pycnidia were placed in the deep-freeze at -20° C. for 30 days.

(ii) Mycelium, pycnidia, and pycnospores

Mycelium, mature pycnidia, and freshly discharged pycnospores, placed on P.S.A., were kept in the deep-freeze at -20° C. until frozen (approx. 2-3 hr.) and transferred to an insulated box containing dry ice (-78° C.) for 3 days. The fungus material was allowed to thaw at 3° C. before being subcultured.

Results

Prior to placing pycnidia in the soil it was found that 100 per cent of the spores germinated. Ninety-five per cent of the pycnospores from pycnidia removed in December 1963 germinated. Subsequently the germination of pycnospores declined to 70 per cent when the last sample was examined in March 1964.

Pycnidia removed in February 1964 were found to be discharging pycnospores.

It appeared that all pycnospores from pycnidia kept at -20° C. for 30 days germinated.

Mycelium, pycnidia, and pycnospores kept at -78° C. for 3 days all produced actively growing cultures.

Discussion

From the growth-rate studies, probably the most significant factor is that the growth of all isolates was similar and that growth was vigorous at 5, 15, and 20° C. These studies agree with Salonen's (41) who found that his isolate P_{30} grew equally well at 7° C. and 22° C.

Extremely low temperatures do not appear to be detrimental for the survival of P. meliloti. This would indicate that it is possible for inoculum, in the form of pycnospores, to be disseminated even under fairly low temperatures. This statement is substantiated by the fact that pycnospores were found to be discharging from pycnidia in mid-winter.

PATHOGENICITY STUDIES

Introduction

The field observations of Markova-Letova (33), Sanford (43), and Salonen (41) indicate that P. meliloti was responsible for the destruction of various legumes. However, infection of host plants with P. meliloti under controlled laboratory conditions by Sanford (43), Cormack (8), and McDonald (34) was not too successful.

Sanford (43) considered that a period of winter dormancy was required for disease development but Cormack (8) obtained infection of alfalfa roots which had not undergone winter dormancy. Goplen (22), in a communication to Dr. Colotelo, stated that, at Saskatoon, infection was not obtained regardless of temperature treatment prior to inoculation.

In the experiments by the above mentioned workers, mycelium was used as inoculum. It was thought, therefore, that infection of host tissue could be obtained using pycnospores as inoculum. As a result, infection studies were carried out on legumes and cereals in which the inoculum consisted of pycnospores, pycnidia, or mycelium.

Fungi are known to excrete products into their growing medium. Some of these substances are toxic to higher plants (36, 2). Cormack in 1934 (8), in his histological studies, states that a toxin was responsible for death of host cells in advance of the fungus.

Therefore, studies were undertaken to determine whether P. meliloti excretes a toxin into its culture medium and to determine the effect of this medium on healthy host plants.

A. Effects of P. meliloti on Host Plants

Materials and Methods

(a) Legumes and cereals grown in P.S.A.

Preliminary experiments indicated that the most favourable medium for growth of seedlings, i.e. for both root and shoot development, was P.S.A. Other media used but found unfavourable included Vogel's (46) and M.Y.A.

Isolates used included P_{10}, P_{15}, P_{20}, P_{30}, P_{40}, and P_{50}. The alfalfa hosts included Medicago falcata, Ladak, Grimm, Cossack, and Ferax; sweet clover varieties Arctic, Cumino, and Erector; cereals included Excelsior barley and Rodney oats.

Alfalfa seeds, variety Ladak, were surface-sterilized by placing them in mercuric chloride (0.1%) for 4 min.

The seeds were washed with sterile water to remove all traces of mercuric chloride and germinated on moistened, sterilized filter paper. Germinated seedlings were transferred aseptically to Petri plates and 250 ml. Erlenmeyer flasks containing P.S.A. The plants were grown at room temperature (approx. 17° C.) in the initial study; however, due to wide fluctuations in temperature a modified growth chamber consisting of an incubator-refrigerator was used.

In this growth chamber the temperature was maintained at 15° C. and a light source of 450 foot candles was supplied by 4-16 inch fluorescent tubes.

The seedlings were grown for 10-14 days and inoculum consisting of spores, pycnidia or mycelium of isolate P_{20} was placed next to the root with care being taken not to injure the root. Roots were taken at various intervals and placed in Rawlin's fixative prior to histological examination. Sampling was continued until the death of the plants.

The above procedure was repeated using mycelium of P_{10}, P_{15}, P_{30}, P_{40}, and P_{50} for Ladak; however, mycelium of P_{20} was used for the infection of Medicago falcata, Ladak, Grimm, Cossack, and Ferax alfalfa; sweet clover varieties Arctic, Cumino, and Erector; and Excelsior barley and Rodney oats. The only departure from the above procedure was that the hulls of Rodney oats were removed for effective surface-sterilization after which the kernels were placed directly into P.S.A.

Histological studies carried out on seedlings followed the same procedure as outlined for pycnidia using safranin and fast green to follow fungus infection.

(b) Alfalfa grown in sterilized soil

Seeds of Ladak alfalfa were surface-sterilized with mercuric chloride (0.1%) and planted in deep Petri plates containing a mixture of soil, sand, and peat (3:2:1). The soil had been previously sterilized at 120° C. for 2 hr., let stand at room temperature for 2 days, and again autoclaved for 2 hr. The resulting seedlings were grown at room temperature and after 2 weeks were inoculated with spores of P_{20}.

To another portion of the soil mixture corn meal was added so that the final mixture consisted of 10 per cent corn meal by weight. This was sterilized by autoclaving and planted with surface-sterilized seeds of Ladak alfalfa. One half of the seedlings were inoculated seven days and the other half 14 days after emergence with pycnidia of P_{15}, P_{20}, and P_{30}. These plants were placed in the modified growth chamber described on page 45.

Uninoculated plants were used as controls.

Plants were examined using histological methods already described.

(c) Greenhouse and field experiments

In the fall of 1963 field-grown alfalfa plants (field collection, variety unknown) were transferred to earthenware crocks and placed in Wisconsin temperature tanks. The temperature was maintained at approximately 10° C. for three days before inoculation. Pycnidia were placed against the root about 5-8 cm. below the crown and held in place by a tuft of absorbent cotton. Pycnidia used for inoculation were obtained from cultures in which the pycnidia were known to contain pycnospores. Plants were examined after two and one-half months.

Seven-month old Grimm alfalfa plants, grown in a deep bed in the greenhouse, were inoculated with pycnidia of P_{15}, P_{20}, P_{30}, and P_{50} in the fall of 1963. Inoculation consisted of placing pycnidia against the root 2-5 cm. below the crown and held in place with a tuft

of cotton. Greenhouse temperature was maintained so that soil temperature was approximately 9.5° C.

One-year old plants of sweet clover varieties Arctic and Cumino were transplanted from the greenhouse to the experimental plots at the University Ellerslie Farm in the spring of 1963. The roots of these plants were inoculated with pycnidia of P_{20} in September of 1963.

The inoculation procedure was different from that previously described in that a plug of tissue (4 mm. dia.) was removed from the root, 7-10 cm. below the crown. Several pycnidia were placed in the cavity and the plug replaced. Control plants were treated in a similar manner but pycnidia were not used.

Plants were examined in May 1964.

Results

The results for all legumes grown in P.S.A. were essentially the same and will be described together.

Mycelium of P. meliloti was found in the cortex as early as four days after inoculation with spores of P_{20}. At this time much of the mycelium appeared to be intercellular (Fig. 13-a,b). Eight days after inoculation the mycelium was chiefly intracellular (Fig. 13-c,d). Ten days after infection the mycelium had ramified through the cortical tissue and reached the endodermis. At this time the root had become visibly brown.

The mycelium, upon reaching the endodermis was temporarily unable to penetrate (Fig. 14-a,b) and heavily massed around the periphery

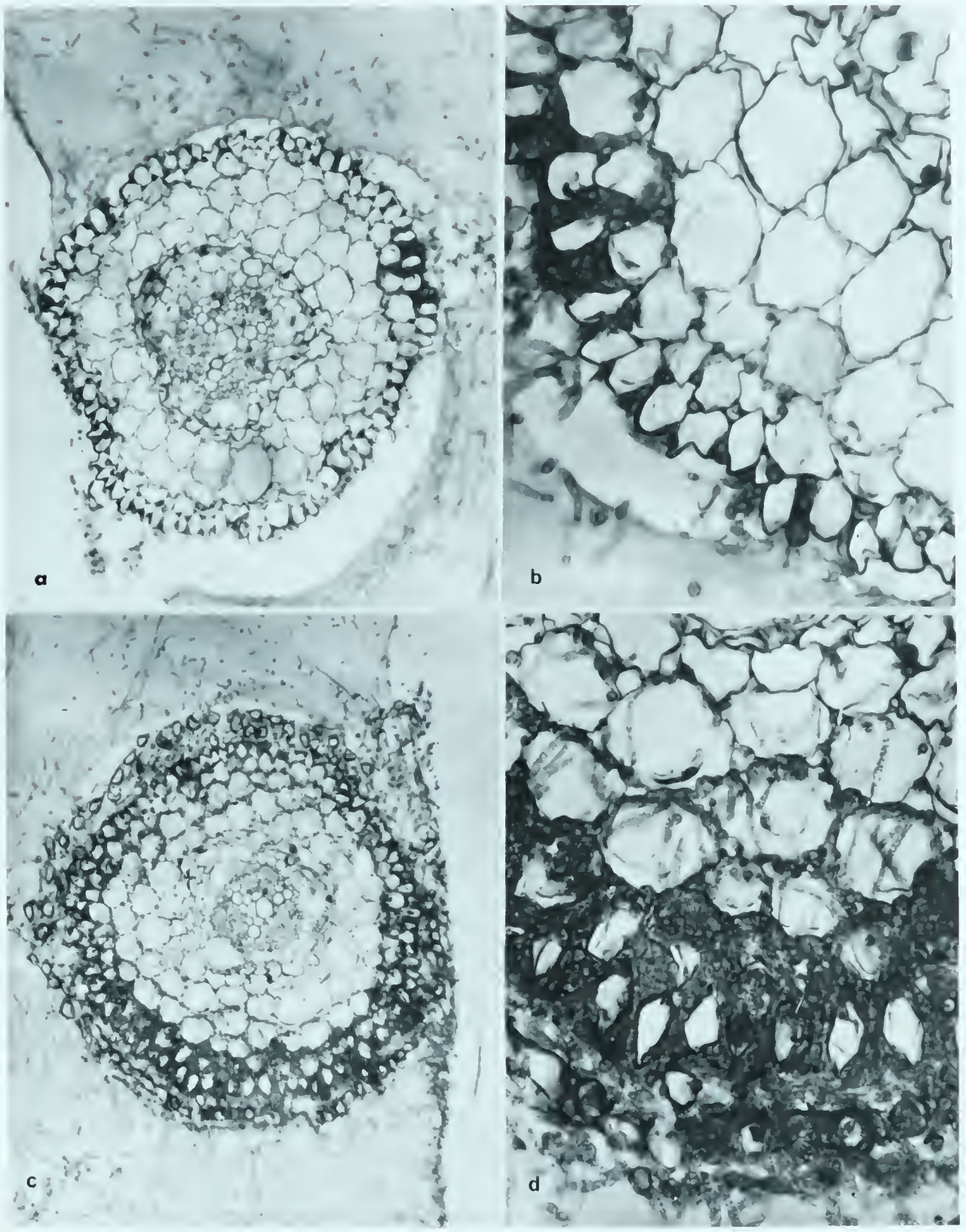


Fig. 13 (a,b,c,d). Infection of seedling roots of Ladak alfalfa by P. meliloti, isolate P_{20}.

(a,b) cross-section of root showing intercellular mycelium four days after inoculation.

(c,d) cross-section of root showing inter- and intracellular mycelium eight days after inoculation.

of the endodermis (Fig. 14-c,d). The endodermal cells appeared to flatten due to the effect of the mycelial mass. At this stage a number of "projections", which stained red with safranin, often appeared along the outer wall of the endodermis adjacent to the mycelial mass (Fig. 14-e) and sometimes penetrated into and through the endodermis. In transverse sections the "projections" were finger-like and in longitudinal sections appeared as two concentric circles within one another.

The endodermis was also crossed or penetrated by another method. Thirty to forty days after inoculation pycnidia formed next to the endodermis (Fig. 14-f), increased in size, and ruptured the endodermis allowing the fungus hyphae to penetrate into the stele. This method of penetrating or crossing the endodermis appeared to be the most common.

The fungus appeared to invade the cortex more readily at the site of the secondary root development. In these regions the hyphae advanced more quickly than in other areas of the cortex.

The appearance of Ladak seedlings grown in P.S.A. 50 days after inoculation with P_{15}, P_{20}, and P_{30} is shown in Figure 15.

With the sweet clover, although the pattern of infection was similar to that of alfalfa, the cortical cells were disorganized to a greater extent in advance of fungus hyphae.

The pathogenesis of P_{50}, isolated from Lupinus arcticus, was much like that of P_{15}, P_{20}, and P_{30} on Ladak alfalfa.

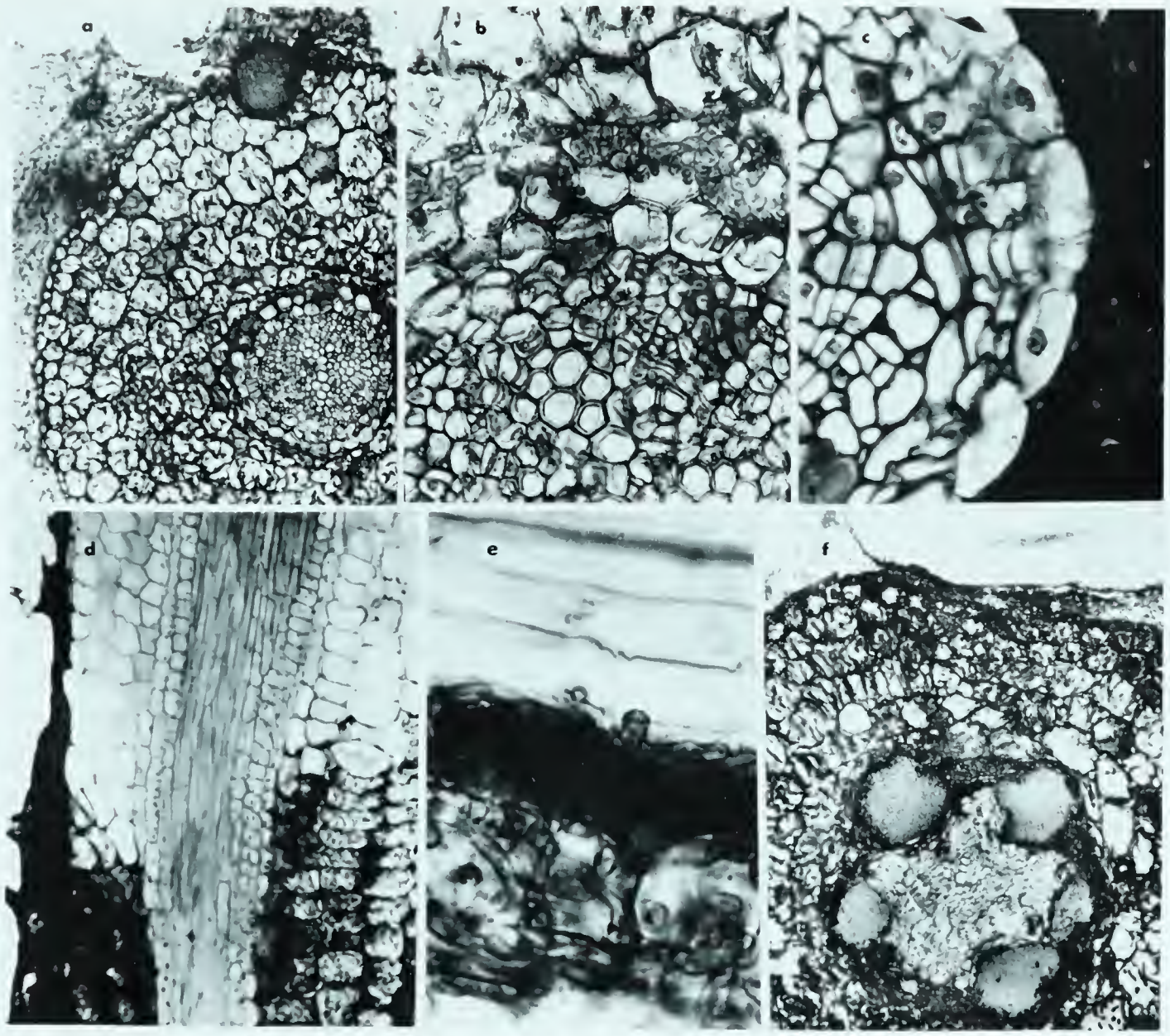


Fig. 14 (a,b,c,d,e,f). Infection of seedling roots of Ladak alfalfa by P. meliloti, isolate P_{20}.

(a) Cross-section of root showing inter- and intracellular mycelium throughout cortex.
(b) Enlarged portion of (a) showing mycelium around periphery of endodermis.
(c) Mycelium heavily massed (black portion) against endodermis.
(d) Longitudinal section of root showing heavy mass of mycelium in cortex.
(e) Cross-section of root showing 'projections' into endodermis.
(f) Cross-section of root showing pycnidia in central portion.

Fig. 15. Control and infected Ladak alfalfa seedlings, grown in P.S.A., 50 days after inoculation. Left to right: control, plants inoculated with P_{15}, P_{20}, and P_{30}, respectively.

The reaction of Ladak alfalfa to isolate P_{40}, isolated from cabbage, was similar to P_{10}, but differed from isolates P_{15}, P_{20}, P_{30}, and P_{50}. Alfalfa roots infected with P_{10} and P_{40} were affected further in advance of fungus hyphae than in the study using other isolates. Cytoplasm of cortical cells stained red and appeared granular prior to penetration of the hyphae. With the other isolates no such reaction occurred in alfalfa, although a similar reaction occurred in cereals infected with isolate P_{20}. This coloration of cytoplasm disappeared shortly after the cortical cells were infected. Projections into the endodermis and pycnidia next to the endodermis were not observed.

Pathogenesis of oats and barley was very similar. Infection of oats and barley was not as rapid as in the case of alfalfa or sweet clover. The mycelium massed around the surface of the root and the cytoplasm of the cortical cells, adjacent to the mycelium, stained pink with safranin and appeared granular (Fig. 16-a,b). The cell walls at this time were brownish in color and later stained heavily with safranin. At this time the affected cells were considered dead. Hyphae then entered these cells. In this manner the hyphae proceeded through the cortex. Upon reaching the endodermis invading hyphae were halted (Fig. 16-c,d), followed by a rapid disorganization of the vascular system. At this stage all the cells of the stele stained red with safranin, several vessels were blocked, and many cells had started to break down.

Projections into the endodermis, and mycelium within the stele, were not observed. Pycnidia were found only on the outer surface of cereal roots.

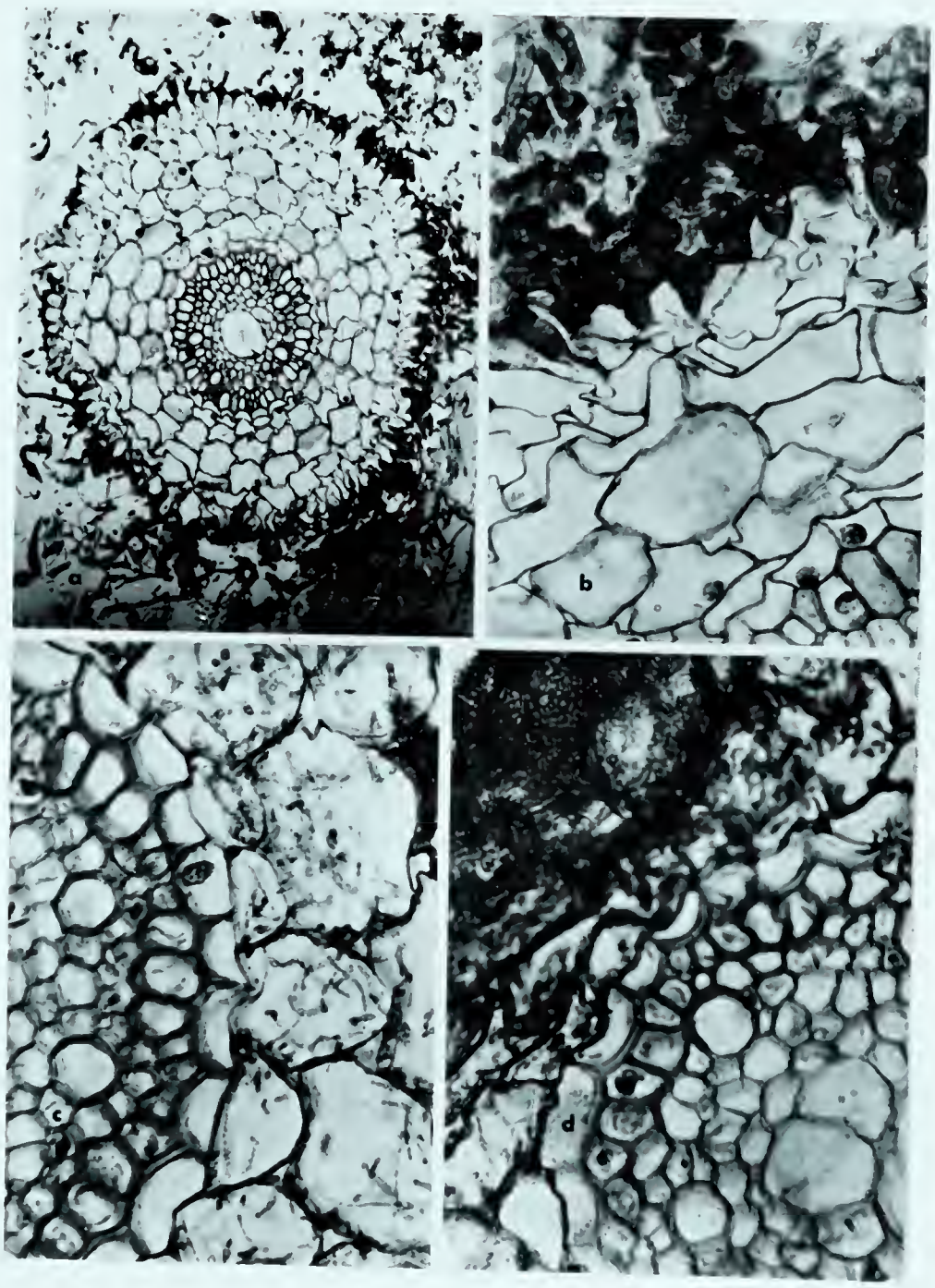


Fig. 16 (a,b,c,d). Infection of seedling roots of Excelsior barley infected by P. meliloti, isolate P_{20}.

(a) Cross-section of root showing mycelium around periphery.
(b) Enlarged portion of (a) showing cortical cells prior to penetration.
(c) Mycelium around periphery of endodermis.
(d) Mycelium heavily massed around endodermis prior to death of root.

Plants grown in sterilized soil (without corn meal) and inoculated with spores of P_{20} were not infected although mycelium was observed in the soil surrounding the plants.

All plants grown in the soil plus corn meal were infected. Externally, there were no visible differences between control and inoculated plants. Pathogenicity of the fungus in alfalfa plants grown in soil plus corn meal, was similar to that of plants grown in P.S.A.

Plants kept in the Wisconsin temperature tank contained brown lesions but no pycnidia were found on inoculated roots. Plants grown in the deep beds showed no infection when examined two months after inoculation.

In field experiments, differences between inoculated and uninoculated plants were not observed.

B. Effects of Toxin(s) on Host Plants

Materials and Methods

Mycelium of P_{20} was grown in shake culture from spores placed in a liquid medium composed of potato extract and sucrose. Seven and ten days after inoculation the liquid from growing cultures was passed through a Seitz bacterial filter to remove fungus mycelium and added to a hot solution of water-agar. To obtain the correct agar concentration a known quantity of agar was autoclaved with a small volume of water and sterilized cultural filtrate added to give a final concentration of 1.5 per cent agar. A portion of the potato extract and sucrose medium which did not contain the fungus, was also incubated and

incorporated with agar, as described, and used as a control. Also a portion of filtrate from 10-day old cultures was autoclaved prior to being incorporated into a medium to determine whether high temperatures would have any effect on the activity of the culture filtrate.

Several alfalfa seeds, variety Ladak, were surface sterilized by placing them in mercuric chloride (0.1%) for 4 min. The seeds were placed on the above "culture-filtrate" medium after they were washed with sterile water to remove all traces of mercuric chloride.

Results

Seedlings grown in 7-day old "culture-filtrate" medium were approximately 1.5 cm. in height and those in the 10-day old "culture-filtrate" medium were less than one cm. in height or even failed to grow after 3 weeks; whereas, seedlings in the non-inoculated "culture-filtrate" medium were 6-8 cm. in height (Fig. 17).

Histological sections of roots from stunted plants showed the cortical tissue to be completely disorganized with only the xylem vessels being recognizable (Fig. 18).

The autoclaved "culture-filtrate" medium was also inhibitory to the growth of alfalfa plants.

Discussion

The experiments with P. meliloti indicate that this fungus is a vigorous parasite on various legumes as well as a good saprophyte.

Fig. 17. Effect of toxin(s) of 10-day old and 7-day old cultural filtrates (left to right) on Ladak alfalfa seedlings. Control extreme right.

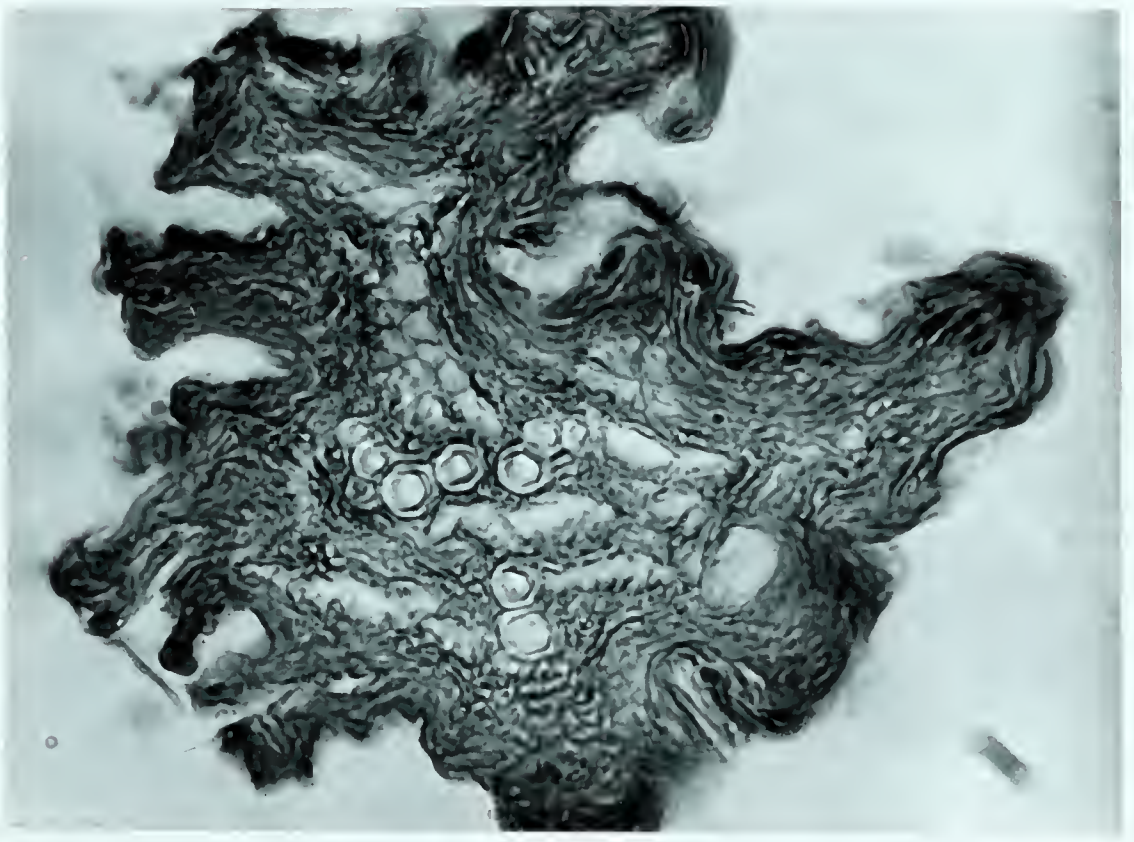


Fig. 18. Section of root of Ladak alfalfa seedling grown in "culture-filtrate" medium showing the effect of toxin(s) produced by P. meliloti.

The experimental results indicate that, in culture, infection of seedlings may be initiated by spores, pycnidia, and mycelium. Cormack (8) observed that mycelium is both intercellular and intracellular. He also found pycnidia within the remains of the cork layer.

Similar projections produced by other fungi in infected hosts have been referred to as callosities by Pearson (37), lignitubers by Fellows (18) and warts by Allen (1). The formation of these projections is thought to be a defense reaction. It is well-known that the casparian strip of the endodermis is involved in regulating the passage of material in and out of the stele. As well as preventing the toxic principle from entering, the suberized wall of the endodermis is thought to delay the penetration of the invading hyphae by depositing a suberin-like substance(s) at the point of penetration. The presence of suberin in the endodermal cell walls of corn is considered by Pearson (37) to act as a barrier to penetration by Gibberella saubinetii. Dickson and Holbart (13) have shown that the resistance of corn to Gibberella saubinetii is due to a well suberized endodermis while the endodermis of susceptible varieties have only patchy areas of suberization.

Since the projections were not observed in oats and barley, it is possible that the endodermis does not react the same as in alfalfa or that an insufficient number of roots were critically examined.

It is interesting to note that M. falcata, generally considered to be resistant to most pathogens of legumes, was found to be very susceptible to P. meliloti. This was also noted for naturally infected field-grown plants in the Yukon.

The reason for not obtaining infection of plants grown in soil without the addition of corn meal is not known. The fact that mycelium is readily observed in the soil mixture, i.e. without corn meal, and that no infection was obtained is not understood at this time. It could be that corn meal provided some factor(s) which promoted the pathogenicity of the fungus. Corn meal had been added by several workers (9, 10, 30) to soil during inoculation experiments with various fungi; however, the reason for its use has not been elaborated.

P. meliloti excretes into the culture medium, substances(s) which are inhibitory to the growth of alfalfa plants. From the histological studies on the pathogenicity of P. meliloti, the death of the cells in advance of the fungus indicated a toxic principle to be active. Therefore, the toxin experiments substantiate the histological observations. Further investigations to determine the nature of the toxin(s) would seem justifiable.

The results of preliminary infection studies in the greenhouse and field inoculated plants were not too encouraging. It would appear that further information is required on factors which would promote infection of older plants. A specific set of environmental conditions may be necessary for successful infection. Little is known about the relationship between the host, effect of other microorganisms, temperature, humidity, and the fungus.

Temperature alone does not appear to account for differences in degree of infection. Soil temperatures recorded at Edmonton and the Dominion Experimental Farm at Mile 1019, Alaska Highway, for the

fall and winter of 1963-64, were quite similar, with the extreme temperatures being recorded at Edmonton. The following spring there was little evidence of damage at Edmonton on inoculated field grown plants; whereas, the plants at the Experimental Farm, Mile 1019, were badly damaged by P. meliloti.

In these studies and those of McDonald (34) it has been found that the prevalence of P. meliloti is greatly reduced during the latter part of the summer. Reasons for this decline are not known. Therefore, a scientific approach to the ecology of the fungus and its relationship to host plants should be made.

SUMMARY

1. A survey for brown root-rot of legumes incited by Plenodomus meliloti, was conducted in central Alberta and in the vicinity of Mile 1019, Alaska Highway, Yukon Territory. The disease damage was slight in Alberta but extensive in the Yukon Territory.

2. Mycelium was found to be generally multinucleate and pycnospores uninucleate. Haploid chromosome number was found to be three.

3. Pycnidia were initiated as a result of simple meristogenous growth. Pycnospores formed on flask-shaped conidiophores were discharged from long beaks.

4. The fungus, P. meliloti, grew well on various culture media.

5. The fungus grew equally well at 5, 10, and 20° C. and under field and laboratory conditions survived extremely low temperatures.

6. P. meliloti was found pathogenic to legume and cereal seedlings grown in culture medium.

7. Infection of mature plants grown under greenhouse and field conditions was not successful.

8. The effects of a fungus toxin(s) were demonstrated.

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APPENDIX

Preparation of Giemsa Stain

Place 3.8 gm. of Giemsa powder (Fisher Scientific) in 125 cc. of glycerine and 375 cc. of methyl alcohol. The phosphate buffer is composed of $Na_2HPO_4 \cdot 7H_2O$, KH_2PO_4 and prepared as follows: 1.0 gm. KH_2PO_4 per 500 cc. of H_2O and 2.0 gm. $Na_2HPO_4 \cdot 7H_2O$ per 500 cc. of H_2O. The KH_2PO_4 was added to the $Na_2HPO_4 \cdot 7H_2O$. For the material under investigation a pH of 6.9 appeared satisfactory for buffer and buffer + stain. Twenty drops of Giemsa stain were used for every 10 cc. of buffer. The final stain is metallic-purple in color.

Preparation of Feulgen Stain

The Feulgen stain was prepared by dissolving 1.0 gm. of basic fuchsin (Fisher Scientific) in 200 cc. of boiling distilled water. This was well shaken and cooled to 50 C. filtered and 30 cc. of 1N HCl and 3.0 gm. of $K_2S_2O_5$ were added. This mixture was placed in an air-tight bottle and kept in the dark for 24 hours, after which 0.5 gm. of carbon (coconut charcoal) were added. The mixture was shaken for 1 min. and filtered. The stain was a straw color and was stored in an air-tight bottle in the dark.

Preparation of Rawlins' Fixative

Absolute ethanol	50	ml.
Distilled water	50	ml.
Formalin 4%	6	ml.
Glacial acetic acid	2.5	ml.